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Basolateral amygdala astrocytes modulate of diabetic neuropathic pain and may be a potential therapeutic target for koumine

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Abstract

Background and Purpose: New remedies are required for the treatment of diabetic neuropathic pain (DNP) due to insufficient efficacy of available therapies. Here, we used chemogenetic approaches combined with in vivo pharmacology to elucidate the role of BLA astrocytes in DNP pathogenesis and provide new insights into DNP therapeutic strategies. Experimental Approach: A streptozotocin-induced DNP model was established. Designer receptors exclusively activated by designer drugs (DREADDs) were used to regulate the activity of astrocytes. Mechanical hyperalgesia was assessed using the electronic von Frey test. Anxiety-like behaviors were detected by open field and elevated plus maze tests. Astrocytic activity was detected by immunofluorescence, and cytokine content was determined by ELISA. Key Results: BLA astrocytes were regulated by DREADDs, and inhibition of BLA astrocytes attenuated mechanical allodynia and anxiety-like behavior in DNP rats. Contrastively, temporary activation of BLA astrocytes induced allodynia without anxious behavior in naive rats. In addition, we found that koumine alleviates mechanical allodynia and anxiety-like behavior in DNP rats, inhibits the activation of BLA astrocytes and suppresses the inflammatory response. Furthermore, persistent activation of BLA astrocytes by chemogenetic mimics chronic pain, and koumine can alleviate its pain hypersensitivity and anxiety-like behavior. Conclusion and Implications: DREADDs bidirectionally regulate the activity of BLA astrocytes, which proves for the first time the role of BLA astrocytes activation in the pathogenesis of DNP and represents a novel therapeutic strategy for DNP. Koumine ameliorated DNP, perhaps by inhibiting the activation of BLA astrocytes and reveal KM as a potential candidate for treating DNP.

Basolateral amygdala astrocytes modulate of diabetic neuropathic pain and may be a potential therapeutic target for koumine

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Number of words in the abstract: 275

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Abbreviations

DNP, diabetic neuropathic pain;

BLA, basolateral amygdala

DREADDs, designer receptors exclusively activated by designer drugs;

KM, koumine;

MWT, mechanical withdrawal threshold;

CNO, clozapine-N-oxide;

STZ, streptozotocin.

What is already known

* Basolateral amygdala (BLA) plays an important role in pain modulation and emotional behavior. BLA astrocytes may be involved in the pathogenesis of pain, but there is no direct evidence.

* Koumine has extensive pharmacological actions, but its brain mechanism in the treatment of diabetic neuropathic pain (DNP) and pain-related negative emotions remain unclear.

What this study adds

* DREADDs bidirectionally regulate the activity of BLA astrocytes, which proves for the first time the role of BLA astrocytes activation in the pathogenesis of DNP.

***** Koumine reduces DNP and anxiety-like behaviors, which is related to inhibiting the activation of BLA astrocytes.

What is the clinical significance

* Activated BLA astrocytes are involved in the pathogenesis of DNP, and specific regulation of BLA astrocytes activity may be a new strategy for the treatment of DNP.

* Koumine may represent a promising drug candidate for DNP.

ABSTRACT

Background and Purpose: Emerging evidence links astrocytes to mechanical nociceptive processing, and the basolateral amygdala (BLA) is a cerebral cortex region that is known to play a key role in pain regulation. However, the association between BLA astrocytes and diabetic neuropathic pain (DNP) pathogenesis remains

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largely unexplored. Here, we used chemogenetic approaches combined with in vivo pharmacology to elucidate the role of BLA astrocytes in DNP pathogenesis and provide new insights into DNP therapeutic strategies.

Experimental Approach: A streptozotocin-induced DNP model was established. Designer receptors exclusively activated by designer drugs (DREADDs) were used to regulate the activity of astrocytes. Mechanical hyperalgesia was assessed using the electronic von Frey test. Anxiety-like behaviors were detected by open field and elevated plus maze tests. Astrocytic activity was detected by immunofluorescence, and cytokine content was determined by ELISA.

Key Results: BLA astrocytes were regulated by DREADDs, and inhibition of BLA astrocytes attenuated mechanical allodynia and pain-related negative emotions in DNP rats. Contrastively, temporary activation of BLA astrocytes induced allodynia without anxious behavior in naive rats. In addition, we found that koumine alleviates mechanical allodynia and anxiety-like behavior in DNP rats, inhibits the activation of BLA astrocytes and suppresses the inflammatory response. Furthermore, persistent activation of BLA astrocytes by chemogenetic mimics chronic pain, and koumine can alleviate its pain hypersensitivity and anxiety-like behavior.

Conclusion and Implications: DREADDs bidirectionally regulate the activity of BLA astrocytes, which proves for the first time the role of BLA astrocytes activation in the pathogenesis of DNP and represents a novel therapeutic strategy for DNP. Koumine ameliorated DNP, perhaps by inhibiting the activation of BLA astrocytes and reveal KM as a potential candidate for treating DNP.

KEYWORDS

Diabetic neuropathic pain, astrocytes, basolateral amygdala, DREADDs, koumine

1. INTRODUCTION

Diabetic neuropathic pain (DNP), a common chronic complication in diabetes, is recognized as one of the most difficult types of pain to treat (Peltier et al., 2014; Alam et al., 2020). It has long been thought that neuronal hyperexcitability and increased synaptic transmission contribute to sensitization of the central nervous system, which promotes the initiation and maintenance of neuropathic pain (Campbell and Meyer, 2006). Neuron-targeting drugs are the main treatment for neuropathic pain, but they are limited by the lack of obvious efficacy and serious side effects (Rosenberger et al., 2020; Finnerup et al., 2021). In fact, it was recently discovered that the opioid analgesic morphine significantly prolonged the duration of hypersensitivity to pain in rats with neuropathic pain (Grace et al., 2016). This problematic feature of the current DNP therapeutic strategies highlights the urgent need to advance our understanding of the mechanism underlying DNP and to identify novel therapeutic targets.

In recent years, the role of astrocytes in neuropathic pain has gradually attracted attention and the focus has gradually shifted from the spinal cord level to the pain-related brain matrix above the spinal cord. (Ji et al., 2019; Tang et al., 2021). Basolateral amygdala (BLA) is an important component of the limbic system, which plays a key regulatory role in pain modulation and emotional disorders (such as fear, anxiety and depression) (Adhikari et al., 2015; Sah, 2017; Hartley et al., 2019, 2019). Even though it has been reported that BLA astrocytes are involved in the development of pain, there is no direct evidence of the role of BLA astrocytes in the pathogenesis of DNP due to the lack of tools for direct and precise regulation of astrocytes. With the advent of chemogenetic tools, designer receptors exclusively activated by designer drugs (DREADDs) has provided the possibility to specifically regulate astrocyte activity in vivo(Shen et al., 2021). In previous studies, we used DREADDs to specifically regulate the activity of motor cortex (MCx) astrocytes, and found that the activation of MCx astrocytes was involved in the exacerbation of neuropathic pain in rats(Lu et al., 2021). Therefore, this study aimed to specifically regulate the activity of astrocytes by DREADDs and provide direct evidence for the involvement of BLA astrocytes in the pathogenesis of DNP.

Koumine (KM) is one of the main alkaloids of Gelsemium elegans*Benth* . Due to its suitable biological activities and few side effects, it has attracted increasing attention from researchers. Previous studies have shown that KM can significantly increase pain thresholds in animals with neuropathic pain by inhibiting glial

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cell activation in the spinal dorsal horn, and KM administration does not result in tolerance or dependence and is safe (Jin et al., 2018, 2021; Shoaib et al., 2019). In addition, neuropathic pain is often accompanied by comorbidities like anxiety, and we found that KM has a significant anti-anxiety effect (Chen et al., 2017). However, whether KM can alleviate anxiety-like behaviors caused by chronic pain and its effect on astrocytes in the BLA remain to be clarified.

In the present study, we employed astrocyte-specific expression of DREADDs to clarify the role of BLA astrocytes in the development of DNP and observed the relationship between the anti-DNP effect of KM and the activation of BLA astrocytes. We provide evidence for the first time that targeting BLA astrocytes may be a potential strategy for the treatment of DNP. KM may significantly reduce mechanical pain and anxiety-like behavior in DNP rats by inhibiting BLA astrocyte activation, and may be a potential new drug candidate for DNP treatment.

2. METHODS

2.1 Animals

Male Sprague–Dawley rats (weighing 180-200 g) were obtained from the Laboratory Animal Center of Fujian Medical University (license no. SCXK (Min) 2016-0002, Fujian, China). All animals were housed in a temperature- and humidity-controlled environment on a 12-hour light/dark cycle with free access to food and water. Before the experimental procedures, animals were randomly allocated into different groups. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Fujian Medical University (Fujian, China) in accordance with the animal care guidelines of NIH (Bethesda, MD, USA). All animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

2.2 Drugs

KM (molecular formula: $C_{20}H_{22}N_{2O}$; molecular weight: 306.1804; PubChem CID: 91895267; purity >99%, HPLC) was isolated from Gelsemium elegans *Benth* . via pH-zone-refining countercurrent chromatography, as described previously (Su et al., 2011); gabapentin (1201301) and streptozotocin (STZ; S0130) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3 Induction ofdiabetic neuropathic pain and assessment

DNP can be readily induced by the systemic administration of STZ (Schnedl et al., 1994). In brief, rats were fasted overnight and then administered an intraperitoneal (i.p.) injection of 70 mg/kg fresh STZ dissolved in 0.1 M citrate buffer solution (pH 4.5) (Courteix et al., 1993). Animals in the control group received an equal volume of citrate buffer. The blood glucose levels from the tail vein were assessed using a One Touch Ultra Easy glucometer (Life Scan Inc., Milpitas, CA) 72 hours after STZ injection. Rats with blood glucose levels [?]16.7 mmol/L were considered diabetic and subsequently included in the study (Jiang et al., 2019). Diabetic rats with neuropathic pain were defined according to the ratio of the 21st-day mechanical withdrawal threshold (MWT) to the baseline MWT < 0.8 as determined by a von Frey test (Fox et al., 1999).

2.4 Mechanical allodynia test

Mechanical allodynia in the diabetic rats was assessed as previously described (Lu et al., 2021). Briefly, rats were individually placed in plexiglass enclosures (28×22×18 cm) with a wire mesh bottom and allowed to acclimate for at least 30 minutes. Using a commercial electronic von Frey apparatus (model 2390, IITC Life Science Inc., Woodland Hills, CA, USA), pressure was applied to the median plantar surface of the right hind paw by a von Frey fiber with a maximum pressure of 55 g. A positive response was defined as an induced pain response (sudden withdrawal, shaking or licking of the hind paw) from the pressure of a filament, and the number on the liquid crystal display was then recorded. The test was performed at intervals of at least 5 minutes to eliminate interference from a previous stimulation. The test continued until three MWT readings were collected after the first change in response. The MWT for each subject was considered the mean of the

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3 collected values. To minimize animal discomfort, mechanical nociceptive thresholds were tested in only one hind paw.

2.5 Open field test

The open field test is a common method for assessing anxiety-like behavior in rats. The apparatus was a 100 cm × 100 cm × 50 cm box (Shanghai Mobiledatum Information Technology Co., Shanghai, China) in a quiet and 30 lux illuminated room. Each rat was placed in the central area and video-recorded for 5 minutes. The total distance traveled in the field and the distance traveled in the central area were measured using SMART software (RRID:SCR_002852, version 3.0). The field was cleaned with 75% ethanol after each test to remove olfactory cues from the apparatus.

2.6 Elevated plus maze test

The elevated plus maze test was performed on the day after the open field test. The apparatus was placed 50 cm above the floor in a quiet and 30 lux illuminated room. It consists of two open arms (40 × 10 cm; no sidewalls; anxiety zone) and two closed arms (sidewalls 10 cm in height; no anxiety zone) connected by a central platform (10 × 10 cm). Each rat was placed alone in the center of the maze with its head toward an open arm. Over the 5-minute session, the time spent in each arm and the frequency of entries into the open arms were determined by video tracking with SMART software (RRID:SCR_002852, version 3.0). The maze was cleaned with 75% ethanol after each test to remove olfactory cues from the apparatus.

2.7 Designer receptors exclusively activated by designer drugs (DREADDs)

We utilized DREADDs as a chemogenetic tool to activate BLA astrocytes. DREADDs receptors are engineered muscarinic receptors that are no longer sensitive to any native ligands, such as acetylcholine, but can be activated by the inert small molecule ligand clozapine-N-oxide (CNO). CNO was purchased from LKT Laboratories, Co., Ltd. (C4759; St. Paul MN, USA). AAV was purchased from Shanghai Taitool Bioscience Co., Ltd. (Shanghai, China). In this study, AAV-gfaABC1D-hM4Di(Gi)-EGFP-WPRE-pA (gfaABC1D-M4-EGFP) was used to specifically inhibit BLA astrocytic activity, and AAV-gfaABC1D-hM3Dq(Gq)-EGFP-WPRE-pA (gfaABC1D-M3-EGFP) was used to specifically activate BLA astrocytes.

2.8 Stereotactic virus injection

Rats were anesthetized with isoflurane (Shenzhen RWD Life Science Co., Ltd., Shenzhen, China) and placed in an automated stereotactic aperture (RWD 68001). A small incision was made in the head to expose the skull, and a small craniotomy was performed. Rats were injected bilaterally in the BLA (coordinates: AP: –2.6 mm; ML: ± 4.8 mm; DV: –8.4 mm; bregma zero) using a 1-μL glass microsyringe for microinjection. The injection volume (500 nL/hemisphere) and rate (60 nL/minute) were controlled by an injection pump (RWD 68606). After the injection, the microsyringe was left in place for at least 10 minutes to prevent the virus from spreading through the injector track, and the injector was then slowly removed. The incision was closed with disposable sterile sutures and needles; for postoperative care, rats were administered an i.p. injection of benzylpenicillin dissolved in saline. The conditions of the rats were carefully observed until they recovered, and the rats were then placed in their original cages. Behavioral tests were performed 3 weeks after virus injection.

2.9 Immunohistochemical staining

The brains were sectioned at 20 μm on a freezing microtome after perfusion and fixation in 4% paraformaldehyde (PFA). The frozen sections were blocked with 10% donkey serum (v/v) for 1 hour and then incubated with GFAP antibody (1:500, mouse monoclonal; Cell Signaling Technology; Cat# 3670, RRID: AB_561049) at 4 °C for an additional 24 hours. GFAP staining was visualized with Alexa 594-conjugated goat anti-mouse secondary antibody (1:200, EarthOx, LLC, San Francisco, CA, USA) and Alexa 488-conjugated goat anti-mouse secondary antibody (1:200, EarthOx). All images were obtained by fluorescence microscopy (Leica DM2000, Leica, Wetzlar, Germany) with Leica Application Suite-X software (Leica, Version 3.4.1). The immunofluorescent intensity of the positive staining area was included and optimized by a low and high

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threshold setup. To quantify the number of GFAP-positive cells, the fields in the BLA area were randomly selected under a confocal microscope at 20× magnification. The related computer-assisted image analysis (ImageJ Software; RRID:SCR_003070; Version 6.0) was conducted blindly by an investigator.

2.10 Enzyme-linked immunosorbent assay (ELISA)

After the completion of behavioral tests, rats were deeply anesthetized with pentobarbital sodium (30 mg/kg) and then sacrificed by decapitation. Then, BLA tissue was collected and prepared using a grinder and an ultrasonic tissue homogenizer. Tumor necrosis factor (TNF)-α (KE20001, ProteinTech Group, Chicago, IL, USA), interleukin (IL)-1β (KE20005, ProteinTech Group, Chicago, IL, USA), CXCL1 (EK0724, Boster Biological Technology, Pleasanton, CA, USA) and MCP-1 (EK0902, Boster Biological Technology, Pleasanton, CA, USA) were detected using ELISA kits in accordance with the manufacturer's instructions. The optical density (OD) values were assessed with a microplate reader (NK3; Ladsystems, Helsinki, Finland) at 450 nm, and the concentrations of TNF-α, IL-1β, CXCL1 and MCP-1 were calculated according to the standard curve and presented as pg/mg protein in the tissue homogenate.

2.11 Data analysis

Statistical analyses were performed using IBM SPSS Statistics software (IBM SPSS Statistics, RRID:SCR_019096; version 21.0). All data are presented as the mean ± S.E.M. and were statistically analyzed by one-way analysis of variance (ANOVA) or two-way repeated-measures ANOVA according to the experimental protocol, followed by unpaired t tests and least significant difference (LSD) or Dunnett's T3 post hoc tests, as appropriate. Probability values <0.05 were considered statistically significant. Groups subjected to statistical analyses had sample sizes of at least 5 animals per group (n=5), where n = number of independent values. The data and statistical analysis complied with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018).

3. RESULTS

3.1 BLA astrocytes were activated in rats with DNP

Within 3 days following an i.p. injection of STZ, blood glucose levels were significantly higher in the diabetic rats than in the control rats (Fig. 1A). All STZ-treated rats exhibited polydipsia, polyuria and polyphagia. Although food and water intake significantly increased, body weights of the STZ-treated rats were profoundly reduced (Fig. 1B). In addition, mechanical allodynia was consistently present in the diabetic rats, compared with the control rats, 7 days after STZ injection, indicating that the diabetic rats developed mechanical allodynia (Fig. 1C).

Anxiety-like behavior is a common comorbidity of chronic neuropathic pain. Next, we assessed the effect of STZ-induced chronic pain on anxiety-like behavior in rats. In the open field test, the total distance of the rats in the open field and the distance in the central area of the open field differ significantly between DNP rats and controls (Fig. 1E-1G). In the elevated plus maze test, we observed DNP rats significantly reduced the number of entries into the open arms and the time spent in the open arms compared to controls (Fig. 1H-1J).

To investigate the dynamic changes in astrocytes in the BLA of the diabetic rats that displayed tactile allodynia, BLA tissues were taken by perfusion, and immunofluorescence histochemical staining was performed. The distribution of astrocytes in the control, day 3 and day 7 groups was reduced, and the immunofluorescence intensity was weak. However, 14 days after STZ injection, we observed a large number of GFAP-labeled astrocytes in the BLA, and the intensity of immunofluorescence was markedly enhanced, reaching a peak at 21 days and lasting at least 28 days (Fig. 1K-1M). The significantly increased expression of GFAP in the BLA after the development of DNP suggests a potential role of BLA astrocytes in the pathogenesis of DNP.

3.2 Short-term DREADD specifically inhibit BLA astrocytes directly reverses mechanical pain and anxiety-like behaviors in DNP rats.

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To investigate the regulation of BLA astrocytes on mechanical allodynia in the rats with DNP, we applied DREADD to manipulate BLA astrocytes. First, the rats were intraperitoneally injected with STZ to induce DNP. Then, we bilaterally injected the gfaABC1D-M4-EGFP virus into the BLA to inhibit BLA astrocytic activity. Three weeks after the gfaABC1D-M4-EGFP virus injection, the rats were exposed to a single dose of CNO, and pain was assessed over a 6-hour time course after CNO administration (Fig. 2A). We observed that mechanical allodynia was rapidly reversed within 1 hour, peaked at 3 hours and remained for 5 hours after CNO treatment in the DNP+gfaABC1D-M4-EGFP group. To test whether these effects were selectively mediated by CNO activation of M4-DREADD, we applied either saline to gfaABC1D-M4-expressing astrocytes or CNO in the rats where gfaABC1D-M4 expression was absent. In both cases, we observed no relief in mechanical allodynia (Fig. 2B). Compared with the MWT in the DNP+gfaABC1D-EGFP group, the MWT in the group of DNP rats expressing gfaABC1D-M4-EGFP was markedly increased by CNO (Fig. 2C).

Next, we used open field and elevated plus maze tests to observe the effect of inhibiting BLA astrocytes on anxiety-like behaviors in DNP rats. After intraperitoneal injection of CNO (1 mg/kg), there was no significant change in the total distance of the rats in the STZ+gfaABC1D-EGFP and STZ+gfaABC1D-M4-EGFP groups in the open field. However, compared with the STZ+gfaABC1D-EGFP group, the movement distance of the STZ+gfaABC1D-M4-EGFP group in the central area of the open field was significantly increased (Fig. 2D-2F). The elevated plus maze test showed that after CNO was administered, compared with the STZ+gfaABC1D-EGFP group, the STZ+gfaABC1D-M4-EGFP group significantly increased the activity time of the rats in the open arm, and the exploration frequency of entering the open arm was also significantly increased (Fig. 2G-2I).

Then, we detected the expression of GFAP in the BLA to determine the effects of CNO-induced gfaABC1D-M4-EGFP receptor activation on astrocyte activity. As expected, CNO treatment significantly attenuated GFAP fluorescence (Fig. 2J-2K) and the number of GFAP-positive cells (Fig. 2L) in the DNP rats expressing gfaABC1D-M4-EGFP compared with the DNP rats expressing gfaABC1D-EGFP.

3.3 Short-term DREADD specifically activate BLA astrocytes induces mechanical pain but not anxiety-like behavior in naive rats.

To further investigate whether the activation of BLA astrocytes was sufficient to cause mechanical allodynia, we activated BLA astrocytes in naive rats with the gfaABC1D-M3-EGFP virus (Fig. 3A). We observed that in the gfaABC1D-M3-EGFP group, mechanical allodynia was gradually induced, peaked at 2 hours after CNO administration, and then returned to normal 6 hours after CNO treatment. However, no significant changes in MWT were found in the gfaABC1D-M3-EGFP group treated with normal saline (Fig. 3B). CNO caused the MWT to significantly decrease in the gfaABC1D-M3-EGFP group compared with the gfaABC1D-EGFP group (Fig. 3C).

Next, we observed the effects of chemical genetic activation of BLA astrocytes on the behavioral performance of rats in open field and elevated cross maze. The results of open field test showed that after intraperitoneal administration of CNO (0.5 mg/kg), rats transfected with gfaABC1D-EGFP or gfaABC1D-M3-EGFP virus had similar trajectories in the open field (Fig. 3D), showing no significant difference in the total distance (Fig. 3E) and the central motor distance (Fig. 3F). Results from the elevated plus maze also showed that after CNO administration, the activity trajectories of the rats transfected with gfaABC1D-EGFP or gfaABC1D-M3-EGFP in the elevated cross maze were similar (Fig. 3G), which showed that there was no significant difference in the activity time in the open arm (Fig. 3H) and the number of explorations into the open arm (Fig. 3I).

Then, we assessed the expression of astrocytes in the BLA to prove that CNO specifically activated gfaABC1D-M3-EGFP-infected cells. We found that CNO significantly enhanced astrocyte expression in the gfaABC1D-M3-EGFP group compared with the gfaABC1D-EGFP group (Fig. 3J-3L). Based on these findings, BLA astrocytes may be an integral part of DNP pathogenesis.

3.4 Koumine reduced mechanical allodynia and anxiety-like behavior in the context of DNP

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Previous studies have shown that the Gelsemium alkaloid KM possesses analgesic activities (Xiong et al., 2017), and we further evaluated the efficacy of intragastric (i.g.) administration of KM on STZ-induced mechanical hyperalgesia. Rats received either STZ or buffer injection. KM (0.28, 1.4, and 7 mg/kg) or vehicle was administered for 7 consecutive days from day 22. Behavioral tests were performed on days 7, 14, 21, and 28 at 1 hour after KM administration (Fig. 4A). Mechanical hypersensitivity was consistently present in the diabetic rats at least 4 weeks after STZ injection, indicated by a reduction in mechanical nociceptive threshold compared with their basal values or those of the control rats. Consecutive treatment of the DNP rats with KM for 7 days increased the withdrawal threshold to mechanical stimuli, although the threshold did not fully recover to the level of the control rats (Fig. 4B).

Anxiety and chronic pain are often comorbid. Next, we sought to evaluate the effect of KM on the anxiety-like behavior in the open field and elevated plus maze tests resulting from DNP (Fig. 5A). In the open field test, we observed that the DNP rats showed both decreased total distance in the open field and number of entries in the central quadrant compared to the control animals. Importantly, after 7 days of KM administration, the total distance traveled in the open field was not significantly changed, but the distance traveled in the central area of the open field was significantly increased (Fig. 5B-5D). In the elevated plus maze test, we found that the DNP rats showed both decreased time and number of entries in the open arms compared to the control rats. KM treatment increased the time spent and the number of entries into the open arms (Fig. 5E-5G). Based on these results, KM effectively improved mechanical hyperalgesia and pain-related negative emotions in the DNP rats.

3.5 Koumine inhibits DNP-induced BLA astrocyte activation and ameliorates the inflammatory response

Given the role of BLA astrocytes in the development of pain hypersensitivity, we sought to examine whether the observed anti-allodynic effects of KM in the diabetic rats could occur by inhibiting the activation of BLA astrocytes. Immunofluorescence staining of the BLA showed that there was an increase proliferation and hypertrophy of astrocytes in the BLA, indicating that the activation of astrocytes was evidently increased in the DNP rats; however, treatment with KM (0.28, 1.4, and 7.0 mg/kg) attenuated the upregulation of GFAP in the diabetic rats (Fig. 6A-6C).

A dramatic increase in inflammatory cytokine levels is one of the major features of astroglial activation. Previous studies have reported that the release of inflammatory cytokines from active astrocytes is the primary cause of DNP (Ji et al., 2014). We examined the expression of proinflammatory cytokines in BLA tissue to investigate whether KM affected the inflammatory response caused by BLA astrocyte activation. ELISA results showed that TNF-α, IL-1β, CXCL1 and MCP-1 levels were markedly increased in the BLA of the DNP rats compared to those in the BLA of the control rats; however, this upregulation was reversed by KM (Fig. 7A-7D). Taken together, these results suggested that KM substantially inhibited DNP-induced BLA astrocyte activation and suppressed the astrocyte activation-induced inflammatory response.

3.6 KM ameliorates chronic hyperalgesia and anxiety-like behavior caused by prolonged activation of BLA astrocytes

Since a single injection of CNO activated BLA astrocytes and instantly induced hyperalgesia, we next investigated whether prolonged activation of BLA astrocytes can induce persistent pain, mimicking the development of DNP. On this basis, we observed whether this pharmacogenetic-induced chronic hyperalgesia can also be alleviated by KM administration.

To this end, we induced repetitive pharmacogenetic activation of BLA astrocytes by daily i.p. injections of CNO for a week in naive rats with bilateral injection of gfaABC1D-M3-EGFP into the BLA and then treated them with KM (Fig. 8A). We found that daily CNO injections for one week induced a persistent decrease in the MWT in the rats expressing gfaABC1D-M3-EGFP, and KM reversed the mechanical allodynia induced by CNO injection in the rats transfected with gfaABC1D-M3-EGFP in the BLA within 1 hour, indicating that prolonged activation of BLA astrocytes was sufficient to induce the development of chronic pain-like hypersensitivity, which could be blocked by KM (7 mg/kg) (Fig. 8B).

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We further determined whether this prolonged pharmacogenetic activation of BLA astrocytes was sufficient to induce anxiety-like behavior. We found that in the rats transfected with gfaABC1D-M3-EGFP, CNO injections made them less active in the central region in the OF and significantly reduced the number of entries and time spent in the open arms in the EPM; KM reversed these effects of CNO injections in the rats transfected with gfaABC1D-M3-EGFP (Fig. 8C-8H). The immunohistochemical results showed in the BLA of the rats that had BLA astrocytes continuously and specifically activated, the fluorescence intensity of GFAP was significantly enhanced, and the number of GFAP-positive cells was significantly increased. When combined with the KM intervention, BLA astrocytes were still, GFAP fluorescence intensity became weak, and the number of positive cells decreased (Fig. 8I-8K).

Collectively, these results indicated that prolonged activation of BLA astrocytes was sufficient to induce chronic hyperalgesia and anxiety-like behaviors that mimic STZ-induced DNP. After administration of KM, prolonged activation of BLA astrocytes induced a fully developed chronic hyperalgesia, and anxiety-like behaviors were significantly improved, which indicated that BLA astrocytes are one of the important targets of the anti-DNP effects of KM.

4. DISCUSSION

In this study, we used the DREADDs approach as a novel tool to examine the role of BLA astrocytes in DNP. Our results showed that inhibiting the activation of BLA astrocytes attenuated mechanical allodynia and pain-related negative emotions in DNP rats. Contrastively, temporary activation of BLA astrocytes induced allodynia rather than anxious behavior in naive rats. In addition, we found that koumine alleviates mechanical allodynia and anxiety-like behavior in DNP rats, inhibits the activation of BLA astrocytes and suppresses the inflammatory response. Furthermore, sustained activation of BLA astrocytes by chemogenetic mimics chronic pain, and koumine can alleviate its pain hypersensitivity and anxiety-like behavior. This evidence suggests that BLA astrocytes are involved in the regulation of pain and pain-related negative emotions in DNP rats and may be an important target through which KM exerted an anti-DNP effect, all of which may suggest a new target for drug development.

In recent decades, the contribution of astrocytes to pain has received considerable attention (Li et al., 2019). Studies have shown that in chronic pain conditions, spinal cord astrocytes are activated, inducing cell proliferation and hypertrophy, accompanied by functional changes (Donnelly et al., 2020). A previous study identified a correlation between spinal astrocyte activation and DNP (Feldman EL et al., 2019). In terms of mechanical allodynia, Liao et al. highlighted that spinal astrocyte activation promoted the development of mechanical allodynia in rats with DNP (Liao et al., 2011). Additionally, numerous studies have shown that inhibiting spinal astrocyte activation can moderate diabetes-induced mechanical allodynia (Nakagawa and Kaneko, 2010; Dauch et al., 2012; Zuo et al., 2015). These studies all support the notion that astrocyte activation is an integral part of neuropathic pain pathogenesis. Although the role of astrocytes in pain modulation is well established, most studies have focused on astrocytic reactions in the spinal cord, whereas a supraspinal understanding of the correlation between astrocytes and neuropathy is still limited.

The amygdala is an important component of the limbic system and plays a key regulatory role in pain sensation and pain emotion (Hua et al., 2020). The BLA, as the main afferent nucleus in the amygdala, has extensive fiber projections to many regions, such as the cerebral cortex, thalamus and brainstem; the BLA can transmit and integrate the emotional and cognitive components of pain from higher brain regions, such as the cortex and thalamus, and subsequently transmits this information to the amygdala complex, where it is combined with sensory components of pain to capture the complete experience of pain perception (Thompson and Neugebauer, 2017). According to previous literature, in a state of chronic neuropathic pain, the activation of brain BLA neurons is involved in the coding of pain, and plastic changes in pain-related neurons are manifested as abnormally increased excitability (Kang et al., 2021). Chemical damage to the bilateral BLA can significantly reduce neuropathic pain-like behaviors in rats, resulting in significant analgesic effects (Li et al., 2013). Furthermore, there is increasing evidence that the BLA is closely associated with negative emotions (Sah, 2017) and cognitive changes (Sun et al., 2019; Zamyad et al., 2021). At the same time, a recent study showed that amygdala astrocytes are significantly activated during the development of

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neuropathic pain (Sagalajev et al., 2018), all of which suggests a potential regulatory role of BLA astrocytes in the pathogenesis of DNP.

To gain insight into the regulatory role of BLA astrocytes in DNP, specific and precise regulation of BLA astrocyte activity in natural physiological and pathological environments is needed. In this study, we employed DREADDs approach to specifically manipulate BLA astrocytes because, compared with traditional pharmacological methods, DREADDs have a strong targeting selectivity, an accurate localization, a lack of neuronal off-target effects and noninvasiveness, which makes it a decisive technique for in vivo exploration of the physiological and pathological effects of astrocytes (Roth, 2016; Pickering and Mazarakis, 2021). The gfaABC1D is a common astrocyte-specific promoter in studies involving DREADDs and has been widely used to study the function of astrocytes in vivo. However, it has also been reported that the gfaABC1D promoter may leak into other cells (Taschenberger et al., 2017). This result indicates that it is important to carefully investigate the specificity of the gfaABC1D-M4-EGFP virus. Therefore, our experimental design excluded the effects of gfaABC1D-M4-EGFP virus on neurons or microglia, which is consistent with Griffin's research (Griffin et al., 2019). Our results showed that the green fluorescence of gfaABC1D-M4-EGFP virus was specifically expressed only in BLA astrocytes and not in neurons and other cells, indicating that the gfaABC1D promoter has the specificity of infecting astrocytes (Supplementary materials).

In the present study, we used a gfaABC1D-M4-EGFP virus to suppress BLA astrocyte activity to evaluate whether inhibition of BLA astrocytes might be beneficial for alleviating mechanical hyperalgesia. We observed that after the verified inhibition of BLA astrocytes with CNO, the mechanical pain threshold was significantly increased, which was similar to the results observed by Marcello et al. after pharmacological intervention (Marcello et al., 2013), indicating that inhibiting BLA astrocytes can effectively alleviate mechanical allodynia in rats with DNP. To further evaluate the effects of BLA astrocytes on mechanical allodynia, we injected the gfaABC1D-M3-EGFP virus into the BLA of naive rats. We observed that, after CNO was administered to activate the virus, the mechanical pain threshold of rats was significantly reduced and returned to baseline by the 6th hour, indicating that activation of BLA astrocytes is necessary and sufficient to induce neuropathic pain-like behavior. These data vigorously illustrated the function of BLA astrocytes in the pathogenesis of DNP. In addition, our previous studies showed that MCx astrocytes also play a role in regulating DNP (Lu et al., 2021), suggesting that cerebral astrocytes are expected to be a novel therapeutic target for DNP.

The link between chronic neuropathic pain and negative moods (anxiety, depression and fear) has proven to be increasingly significant since the link is bidirectional, and both act as risk factors for each other (Baliki and Apkarian, 2015). In this study, we observed an obvious anxiety-like behavior in DNP rats, which was specifically manifested as reduced exploration intention and activity ability. Previous studies have shown that chronic pain in rats does not reduce total distance traveled in open field (De Gregorio et al., 2019). However, we found that compared to the control group, the total distance of activity in the open field was significantly reduced in DNP rats, which may be due to chronic pain following hind foot pain and a reluctance to exercise in the open area, thereby reducing the harmful stimulation of the post foot. In addition, we found that the DREADD specific inhibition of the activation of BLA astrocytes in DNP rats could effectively alleviate the anxiety-like behavior of DNP rats, which was similar to Xiao et al 's findings by using optogenetic technology (Xiao et al., 2020). Interestingly, we found that the transient activation of BLA astrocytes by DREADD did not significantly affect the behavior of rats in the open field and elevated cross maze, which may be due to a single injection of CNO activation of BLA astrocytes in naive rats caused by transient pain is not enough to make rats produce negative emotions in the short term.

Increased attention has recently focused on the examination of the analgesic potential of phytoconstituents, and finding active monomers from medicinal plants plays an important role in the development of new drugs (Almeida et al., 2001). Gelsemium, a perennial evergreen entangling vine plant from the family Loganiaceae, contains KM, the most dominant alkaloid in G. elegans Benth, alters variety of biological functions and has great value in the context of new drug development (Zhang and Wang, 2015). Previous studies have shown that KM has marked antinociception in inflammatory and neuropathic pain without inducing antinociceptive

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tolerance (Xu et al., 2012; Xiong et al., 2017). In addition, studies have shown that KM has anti-anxiety properties without inducing adverse neurological effects (Liu et al., 2013; Chen et al., 2017). In line with these findings, we discovered that KM can significantly alleviate DNP and effectively mitigate anxiety-like behavior in DNP rats, suggesting that KM is expected to become a candidate new drug for the treatment of DNP.

DREADDs are chemogenetic tools widely used to remotely control cellular signaling, neuronal activity, and behavior and have emerged as powerful tools with great potential for drug discovery and development (Lee et al., 2014). In this study, we combined DREADD approach with in vivo pharmacology to parse the role of BLA astrocyte in chronic pain, demonstrating that BLA astrocytes are an important target for KM in exerting anti-DNP effects. DREADD were used to continuously activate BLA astrocytes in naive rats to mimic animals in a chronic neuropathic pain model; these rats were then treated with KM to observe the antagonistic effect of KM on the DREADD-induced effects. Similar to the report by Sun et al. (Sun et al., 2020), our data showed that the phenotype of chronic neuropathic pain animals can be simulated by activation of BLA astrocytes in naive animals by CNO for 7 consecutive days, which was specifically manifested in the continuous mechanical pain sensitivity. Unexpectedly, we found that repeated administration of CNO to continuously activate BLA astrocytes also induced anxiety-like behaviors in naive rats, in contrast to the results of short-term activation of BLA astrocytes with a single injection of CNO. The simultaneous administration of KM, however, gradually reversed the chronic neuropathic pain and improved anxiety-like behavior induced by continuous activation of BLA astrocytes. By combining the chemogenetic approach with in vivo pharmacological manipulations, it was further confirmed that BLA astrocytes may be a potential target for DNP treatment.

Astroglial activation is associated with increases in proinflammatory cytokines (Sommer et al., 2018). An increasing number of studies have shown that the proinflammatory cytokines released by activated astrocytes play a crucial regulatory role in neuropathic pain sensitization (Linnerbauer et al., 2020). Many experiments have confirmed that the proinflammatory cytokines TNF-α and IL-1β are involved in the regulation of neuropathic pain (Hung et al., 2017). In addition, in chronic pain conditions, activated astrocytes express a large number of chemokines including MCP-1 and CXCL1, which act on CCR2 and CXCR2 in spinal cord neurons (Zhang et al., 2017). Activated CCR2 and CXCR2 induce the activation of extracellular signal-regulated kinases, which rapidly phosphorylate NMDA receptors, increase excitatory synaptic transmission, and promote central sensitization after nerve injury (Moraes et al., 2020). We observed that the STZ-induced DNP model rats had sustained mechanical hyperalgesia and massive activation of astrocytes in the BLA, accompanied by TNF-α, IL-1β and chemokines CXCL1 and MCP-1 in the BLA. Importantly, we also confirmed that KM can reduce the expression of TNF-α, IL-1β, CXCL1 and MCP-1, which is consistent with previous findings. In general, these results demonstrate that BLA astrocytes not only are transmission intermediaries for neuropathic pain but also actively participate in the production of neuropathic pain, and KM can improve the mechanical hyperalgesia and anxiety-like behaviors of DNP rats by inhibiting the activation of BLA astrocytes.

In conclusion, our current findings highlight for the first time that activation of BLA astrocytes plays an important role in the pathogenesis of DNP, which may be a target for the treatment of DNP. The anti-DNP effect of KM may be related to inhibiting the activation of BLA astrocytes, and reveal KM as a potential candidate for treating pain and pain-related anxiety-like behaviors.

AUTHOR CONTRIBUTIONS

J-SL, LY, JC, F-FX, PC, X-YW, B-JX, Z-HC performed research; J-SL, LY, JC analyzed data; J-SL, LY wrote the first draft of the paper; J-SL, JC and C-XY edited the paper; LC, JY and C-XY conceived and designed the research. All authors reviewed and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for *Design & Analysis* ,*Immunochemistry* and *Animal Experimentation* and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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